CORRELATION TIMES OF SPIN-LABELLED ANALOGS OF ADENOSINE 5'-MONOPHOSPHATE

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ABSTRACT

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CORRELATION TIMES OF SPIN-LABELLED
ANALOGS OF ADENOSINE 5'-MONOPHOSPHATE

Advisor: Dr. Joe Johnson

Thesis dated May 1977

The C-8 spin-labelled derivative of adenosine 5'-monophosphate (AMP) was synthesized by reacting 8-thio-AMP with 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy spin-label. Circular dichroic spectra indicate that substitution at C-8 markedly influences the conformational equilibrium in AMP. Activity studies performed on AMP, 8-bromo-AMP, 8-thio-AMP, and the spin-labelled nucleotide indicate that bulky substituents reduce the affinity of FbPase for AMP derivatives. Through ESR studies, correlation times were calculated for the free spin-label and the spin-labelled nucleotide.

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Special thanks are given to my parents.

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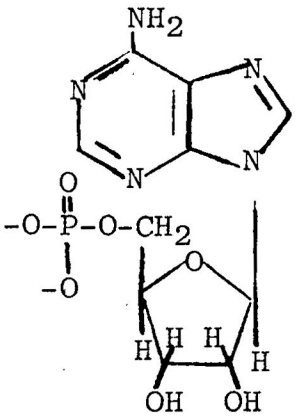
INTRODUCTION

Adenosine 5'-monophosphate (AMP, 1) is a purine nucleotide. It is an inhibitor of the regulatory enzyme, fructose-1,6-bisphosphatase (FbPase). This enzyme plays a key role in the regulation of glycolysis and gluconeogenesis. Besides being a regulatory enzyme which is strongly inhibited by the negative modulator AMP, it has three or more binding sites for AMP. These binding sites are distinct from the substrate binding site(s).



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By using a combination of techniques, i.e., circular dichroism (CD), optical rotary dispersion (ORD), and fluorescence spectroscopy, evidence for an AMP-induced conformational change in the enzyme can be observed. While these techniques can only detect and monitor conformational changes, they can give no quantitative information of the extent of such changes or the distances between various sites on the enzyme. On the other hand, a spin-label probe (non-covalently or covalently

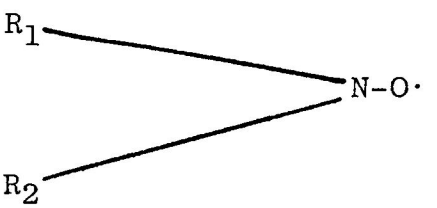
bound) can be used to obtain this type of information. For many years labelling techniques have been used in biological investigations to probe the structure of biological systems and mechanisms of biological reactions. As an example of the former type of application, fluorescent labels on proteins have yielded significant information about conformations in biological molecules. In the latter technique carbon-13 labelling has enabled elucidation of the detailed mechanism of many biological reactions.

It would be desirable if any biological system or molecule could be made paramagnetic in a specific way at will. It would seem a natural extension of the fluorescent label technique to develop "spin-labels" (stable organic free radicals) that could be attached to a specific site on a molecule in a complex system and whose electron spin resonance (ESR) spectra would contain information about the environment of the label. In 1964, H. M. McConnel began a program to design and synthesize such spin-labels and apply them to biological systems.[1]

Electron spin resonance spectroscopy is a physical technique designed to detect species with unpaired electrons.[1] Like other forms of spectroscopy, ESR monitors the net absorption of energy from a radiation field when molecules change their energy state. Associated with the radiation field are oscillating electric and magnetic fields perpendicular to one another. In most forms of spectroscopy it is the electric field component that interacts with the

molecules to cause the change in energy state. For instance, in visible spectroscopy absorption results from transitions of electrons from their ground state to excited states.[1]

An essential property of a spin-label is stability under conditions used in the study of biological molecules. It must be sensitive to its environment--preferably to polarity, acidity, spatial restrictions, and fluidity.[1] The ESR spectra must be relatively simple to interpret. To be generally useful it is necessary that the compounds used as spin-labels have a well understood chemistry that permits synthesis of functional groups for particular purposes. The most successful compounds used as spin-labels to date are the nitroxides of the general structural formula illustrated below.



The ESR spectra of the nitroxide spin-label are sensitive to the rotational correlation time (or tumbling time) of the spin-label. When the spin-label is tumbling freely in solution (with a correlation time of 10^{-11} seconds or less), the spectrum consists of three sharp lines (Fig. 1). If the spin-label is rigidly bound to a protein such that its correlation time is 10^{-8} seconds or longer, the anisotropic electron-electron and electron-nuclear interactions

broaden and displace these lines to give a spectrum similar to Fig. 2.

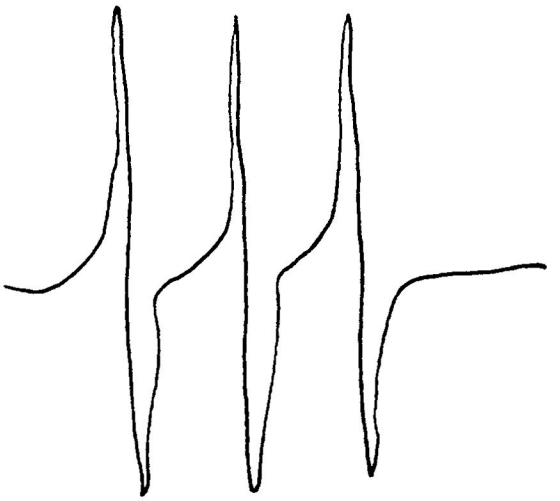


Fig. 1. The ESR spectrum of a nucleotide spin-label (pH 7).

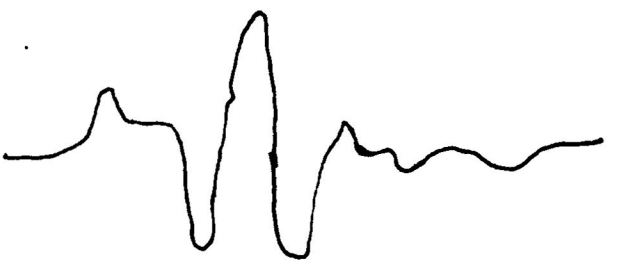


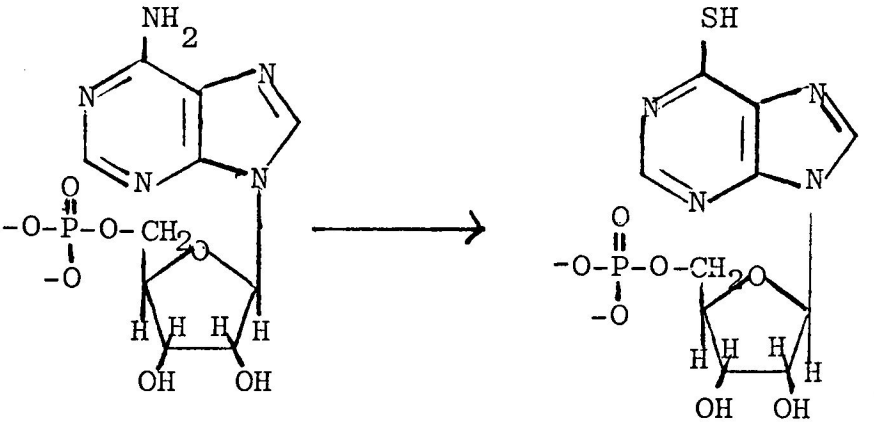
Fig. 2. The ESR spectrum of a nucleotide spin-label bound to an enzyme (pH 7).

The electron spin resonance spectrum of the nitroxide label consists of three lines (assigned $M_I = 1$, $M_I = 0$, and $M_I = -1$, ^{14}N nuclear spin quantum states, the $M_I = 1$ state corresponds to the line at lowest field).[3] The lines are

of unequal widths. The inequality is a measure of the anisotropies of the hyperfine interaction and of the g value.

A spin-label may be attached to the system of interest by a covalent bond or by non-covalent forces such as those involved in enzyme-coenzyme, enzyme-substrate, antibody-hapten, and membrane-steroid interactions. The object of the non-covalent spin-label is to make it structurally as similar as possible to a natural component of a biological system or to a compound that interacts with the system.

A number of spin-labelled coenzymes have been prepared. A nitroxide spin-label having iodacetamide reactivity has been attached to a sulfhydryl group, 3, which had been substituted for the amino group, 2, of adenosine 5'-triphosphate (ATP).[2] Analogs of nicotinamide adenine dinucleotide (NAD, 4), and vitamin B_{12} (5) have also been prepared. The N^6, C-8, and C-2 spin-labelled derivatives of adenosine 3',5'-monophosphate (cAMP, 6) have been synthesized.[3] It thus seemed feasible to investigate the synthesis of certain AMP analogs. The possibility of preparing a spin-labelled AMP seems especially attractive for future biochemical studies.



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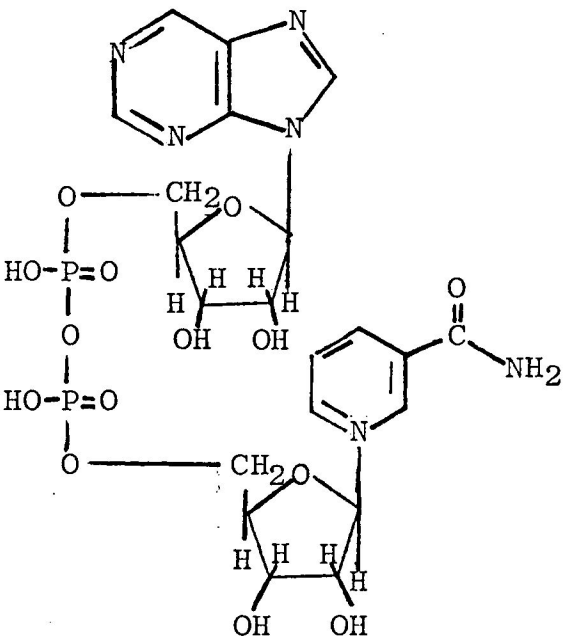

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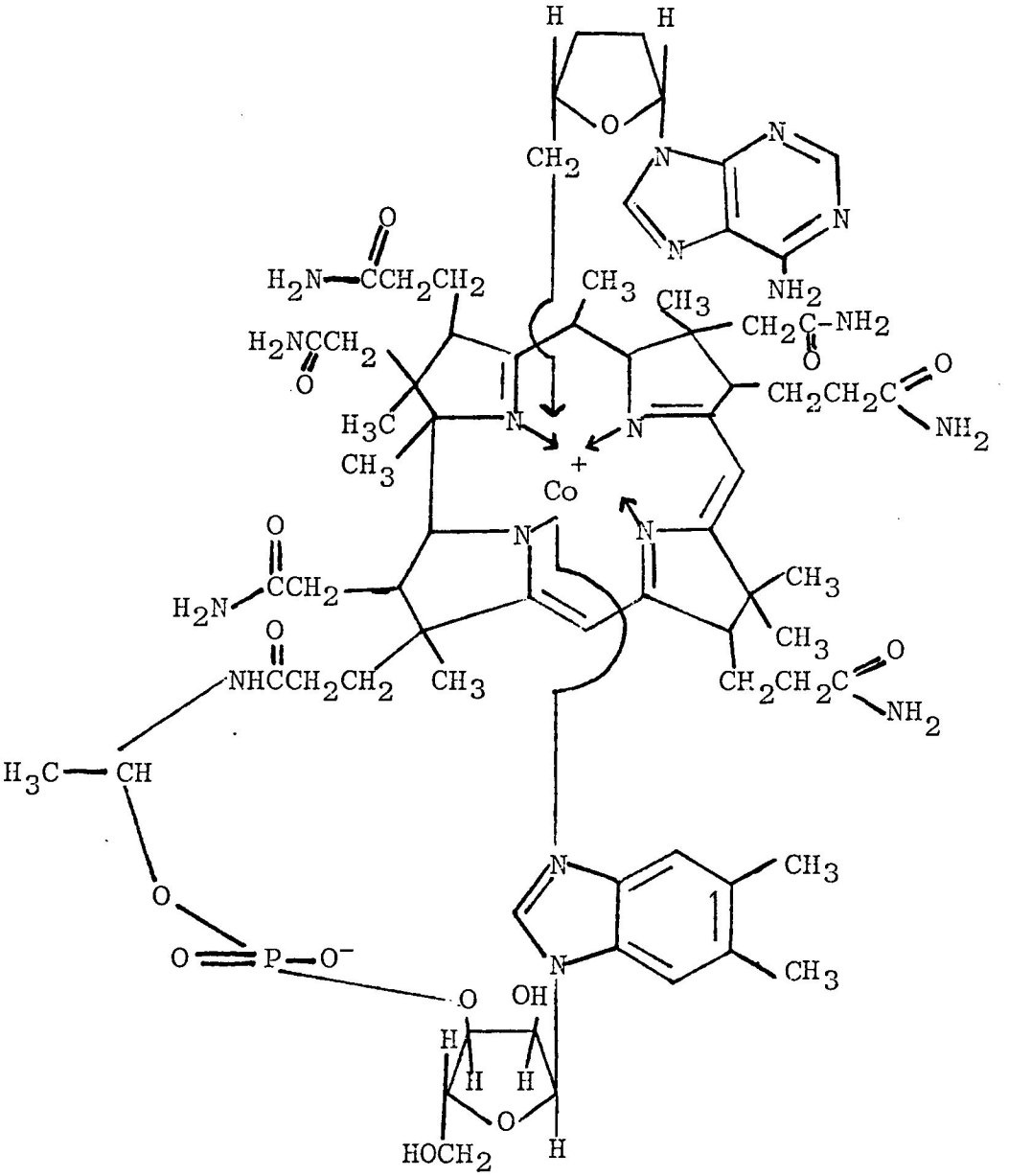
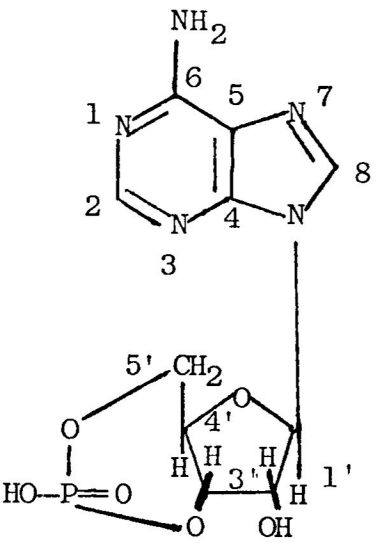


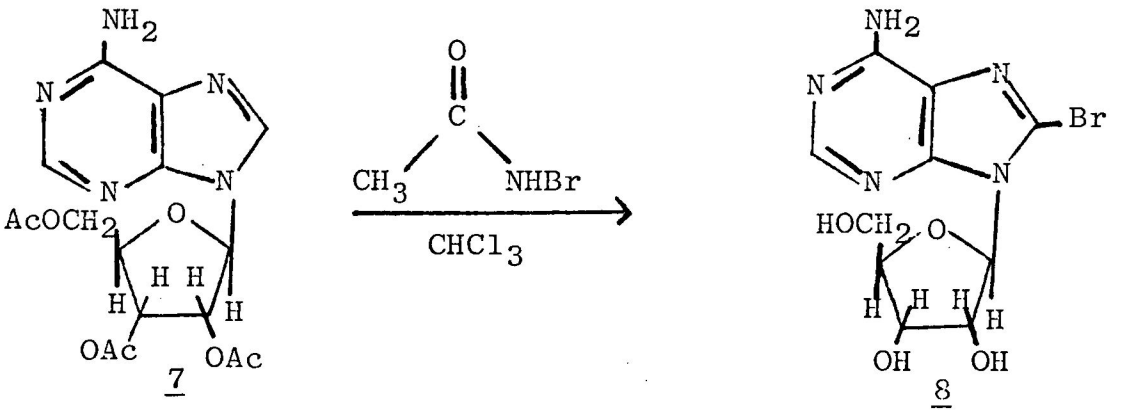


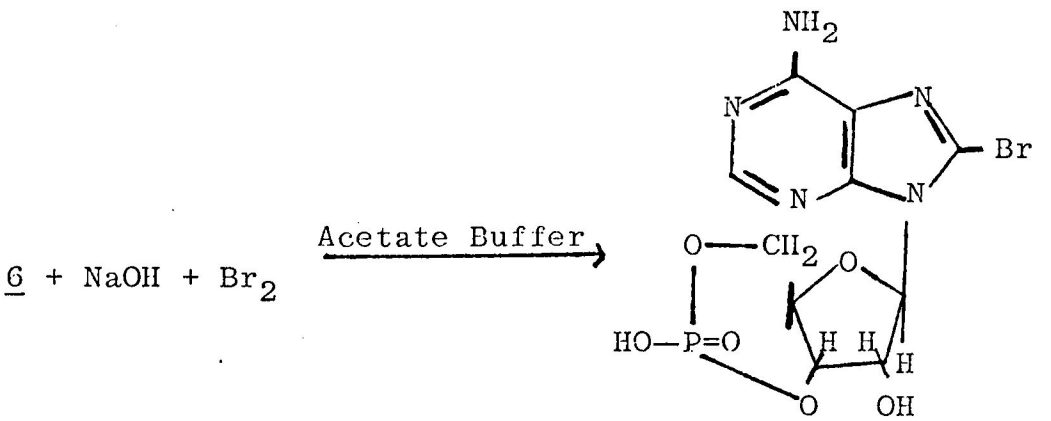
6

Direct halogenation of various pyrimidine ribosides and deoxyribosides has been known to provide the appropriate 5-halogenated pyrimidine nucleoside.[4] Interest in recent years in the biochemistry of nucleic acids has stimulated improved procedures for halogenation. The halogenation of naturally occurring purine nucleosides has, during the last ten years, received more attention.

In 1961 and 1964 Robins and coworkers synthesized several bromopurines and purine nucleosides.[4,5] Bromination of tri-O-acetyl adenosine (7) was accomplished by using N-bromoacetamide in chloroform. Deacetylation of the product gave 8-bromoadenosine (8). The position of the entering bromine was confirmed by acid hydrolysis to the known 8-bromoadenosine and α-ribose.[4] In 1971 Robins and coworkers prepared 8-bromo-cAMP (9) with the use of bromine in acetate buffer (pH 3.9) in the presence of sodium hydroxide.[7] Success in the synthesis of various 8-substituted-2'-

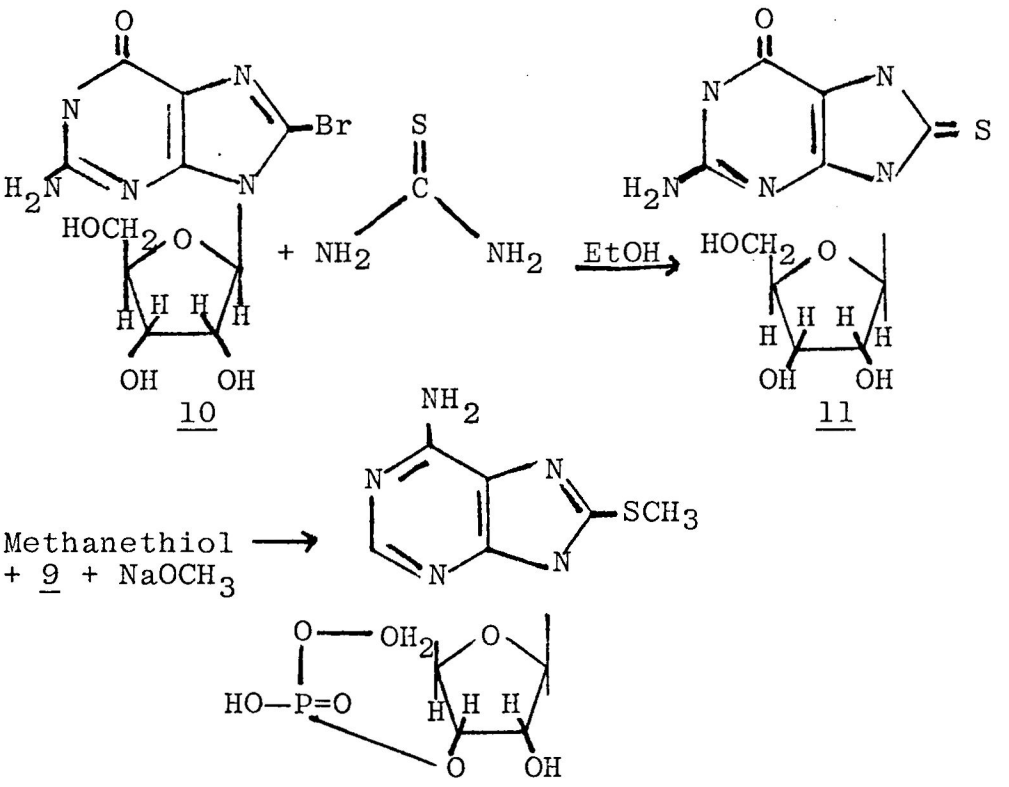
deoxyadenosines suggested that selected nucleophiles might react under relatively mild conditions.[4,8]





The preparation of 8-bromo-AMP provides a useful synthetic intermediate since the bromo group can be replaced by various nucleophilic reagents. For example, 8-bromoguanosine (10) and thiourea in refluxing ethanol readily provides 8-mercaptoguanosine (11).[6] Treatment of 8-bromo-cAMP with sodium methoxide in the presence of methanethiol in methanol gave 8-methylmercapto-cAMP (12).[4] Thiouridine (14), initially prepared by Fox and coworkers,[9] was prepared with liquid

hydrogen sulfide pyridine and cytidine (13).[10] Similar treatment of sodium cytidine 5'-phosphate (15A) cytidine 5'-diphosphate (CDP, 15B), cytidine 5'-triphosphate (CTP, 15C), and CDP-choline (15D) afforded the respective 4-thiouridine phosphate (16).[6] Several cytidine derivatives such as arabinofuranosyl cytosine (17), 3-methylcytidine (18), and 2-thiocytidine (19) also underwent sulfhydrolysis affording respective 4-thio derivatives. Treatment of the 4-amino-2-methyl thiopyrimidine (20) with 2,3,5-tri-O-benzoyl-D-ribosyl pyrimidinum chloride (21) afforded the ribosyl pyrimidinum chloride (22), which on treatment with hydrogen sulfide, gave 2',3',5'-tri-O-benzoyl-2-thiocytidine (23) and 2-thiocytidine (24) by further de-benzoylation.[11]



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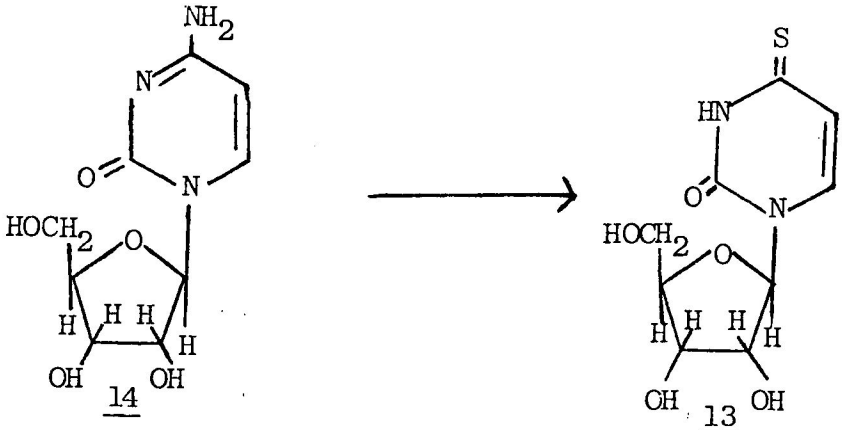

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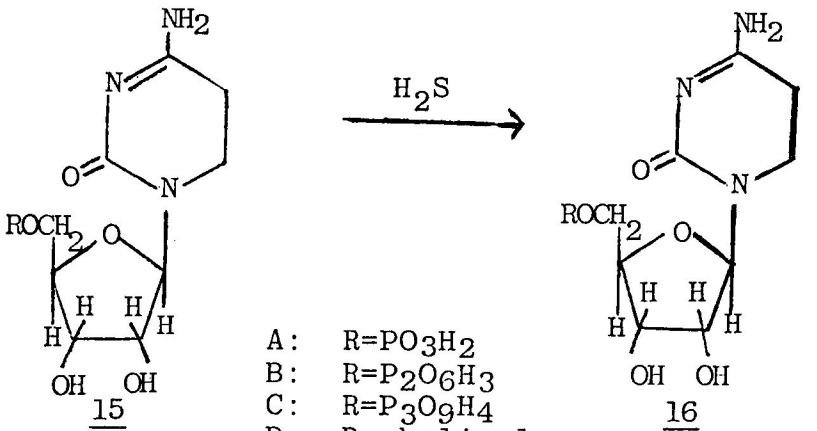
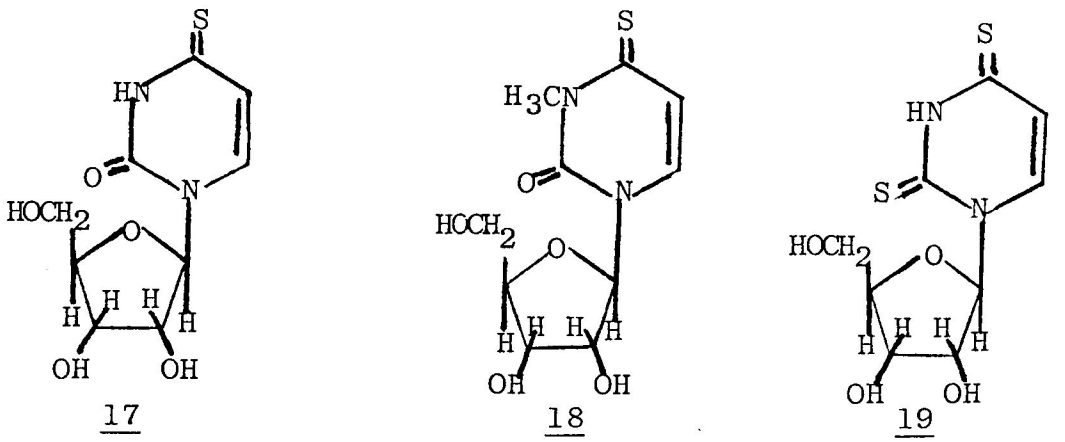


15 16

A: R=PO_3H_2
B: R=$P_2O_6H_3$
C: R=$P_3O_9H_4$
D: R=cholinyl pyrophosphoryl



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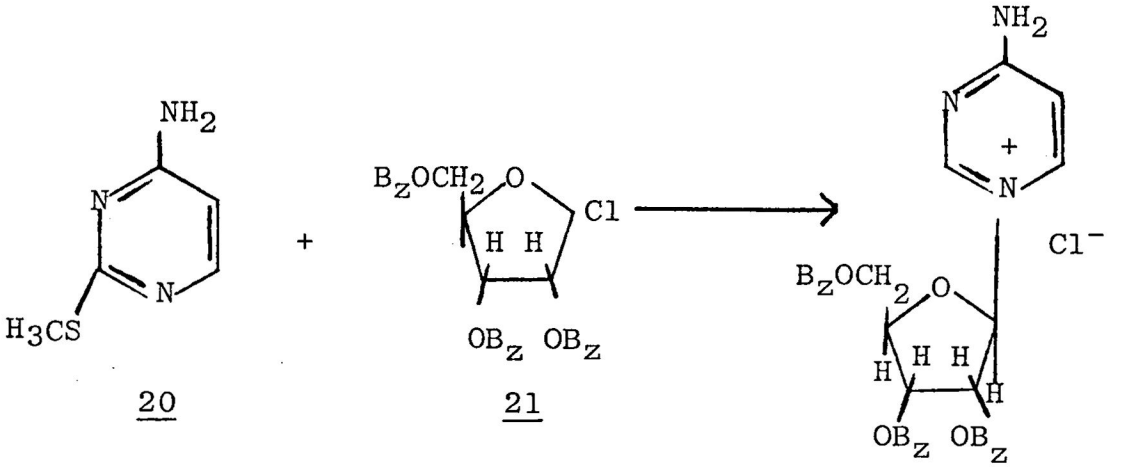

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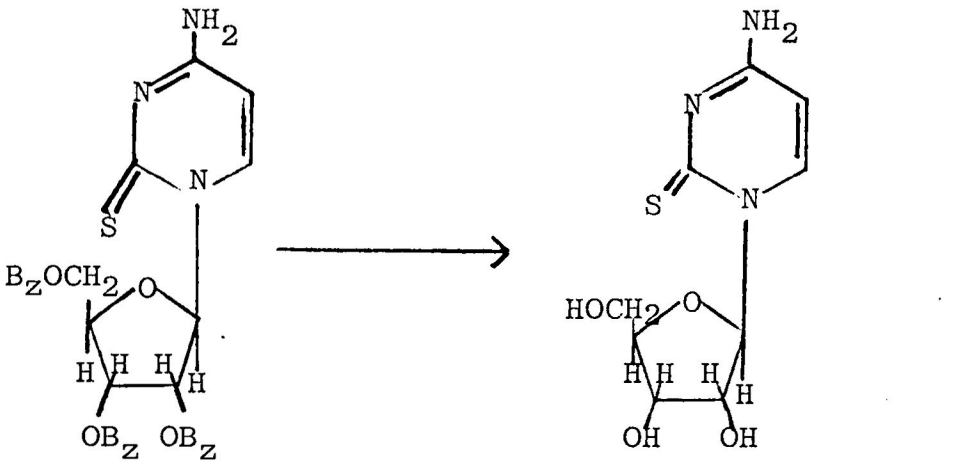
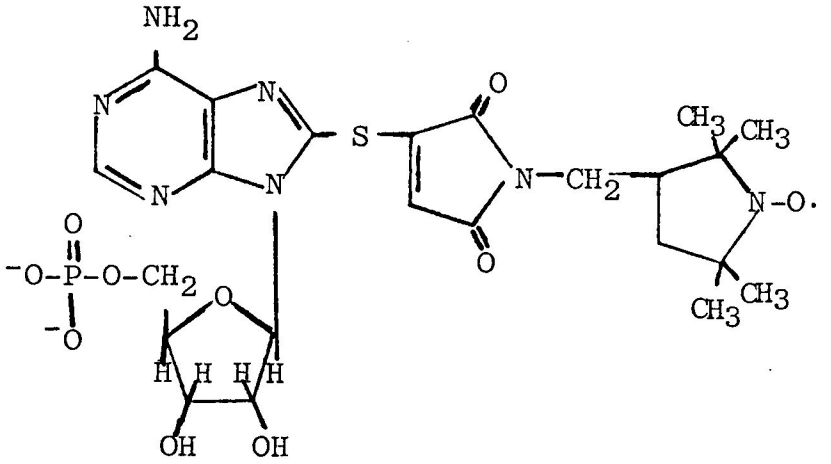


23 24

In the present investigation a spin-labelled AMP derivative, 8-mercapto-3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl adenosine 5'-monophosphate (25), was synthesized. Using the spin-label probe attached to FbPase, the extent of conformational changes induced by the AMP derivative could be obtained from calculations of the

correlation times.



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EXPERIMENTAL

Adenosine 5'-monophosphate (AMP), p-(hydroxymercuri)-benzoic acid (HMBA), sodium salt and hexamethylphosphoramide (HMPA) were purchased from the Aldrich Chemical Company. The 8-bromoadenosine 5'-monophosphate was purchased from the Sigma Chemical Company. The 3-maleimide-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl spin-label was purchased from Eastman Kodak Company. Benzoyl chloride and aqueous ammonia were purchased from the J. T. Baker Company. Sodium thiocyanate was purchased from Fisher Scientific Company.

Infrared (IR) spectra were recorded on a Beckman Infrared-4240 spectrometer and calibrated against polystyrene film. Unless otherwise specified, potassium bromide (KBr) pellets were used for determining infrared spectra. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Associates A-60 spectrometer using deuterated acetone (acetone-d_6). Melting points were made using a Thomas-Hoover melting point apparatus. Ultraviolet (UV) spectra were recorded on a Varian Cary-17 spectrophotometer and a Hitachi Digital 191 spectrophotometer equipped with a N-14 printer and N-23 kinetic timer. Electron spin resonance (ESR) spectra were recorded on a Varian E-3 ESR spectrometer.

Anhydrous Methanol Solution.--Methanol (500 ml) was refluxed over anhydrous calcium chloride for 4 hr. The mixture was distilled slowly at 65^{o} and stored over molecular sieves.

Bromination of Adenosine 5'-Monophosphate.--To a suspension containing adenosine 5'monophosphate (AMP, 1.32 g, 3.5 mm) in anhydrous methanol (2.0 ml), sodium methoxide (0.2166 g, 3.7 mm) was added. To this suspension bromine (0.28 ml, 5.075 mm) dissolved in methanol (55.0 ml) was added. The progress of the reaction was monitored by thin layer chromatography (TLC). Typically the reaction was completed in 48 hr at 65°. Ultraviolet spectra were recorded of the resulting mixture at 264nm. The suspension was separated on a silica gel column and eluted with methanol and water (v/v). Fractions (~5 ml per tube) were collected and stored in a refrigerator freezer for 72 hr. The precipitate formed was tested by TLC and UV spectroscopy for purity. The precipitate was filtered and put on a charcoal (ground) column and eluted with the same solvent as was used in the silica gel chromatography. Solvent was removed by rotary evaporation. The residue was shown to be chromatographically pure. The product was then tested for the presence of inorganic phosphate.

R_f values, melting points, and UV λ_{max} for AMP are 0.59, 205°, and 260nm, and for 8-bromo-AMP are 0.44, 155-7°, and 264nm.

Test for Inorganic Phosphate.--Analysis of inorganic phosphate was performed on solutions containing AMP, 8-bromo-AMP, 8-thio-AMP, and monopotassium phosphate according to the method of Fiske and Subbarrow.[12]

Solutions containing inorganic phosphates turned blue.

8-Mercaptoadenosine 5'-Monophosphate (8-SH-AMP).--To a suspension containing 8-bromo-AMP (100 mg, 2.35 mm) in ethanol (95%, 25 ml) 1-benzoyl-2-thiourea (80 mg, 0.47 mm) was added. This suspension was stirred for 24 hr at 40°. The reaction was monitored by TLC. Infrared and UV spectra were recorded on the resulting product. The product was then tested for the presence of sulfur.

Melting point for 8-mercapto-AMP is 135-140°, and UV λ_{max} is 270 and nm.

Anal. Calcd. for $C_{10}H_{12}N_5O_7PS$: C, 31.83; H, 3.18; N. 18.57; 0, 29.71; P, 8.22; S, 8.49. Found: C, 36.85; H, 4.16; N, 15.24; P, 4.23; S, 6.29.

1-Benzoyl-2-Thiourea.--The compound, 1-benzoyl-2-thiourea, was prepared by the method of Klayman, Shine, and Bower.[13]

Test for Sulfur.--Analysis for sulfur was performed according to the method of J. L. Bailey.[14]

8-Mercapto-3-maleimido-2,2,5,5-tetramethyl-1-pyrrolinyl-oxy Adenosine 5'-Monophosphate.--To a solution containing the nucleotide sample (24.8 mg, 0.066 mm) in ethanol (95%) 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy spin-label (10 mg, 0.066 mm) was added. The mixture was allowed to spin (on magnetic stirrer) at room temperature for 1 hr. Infrared and ESR spectra were taken of the unreacted spin-label and spin-labelled nucleotide in tris-HCl buffer (pH 6.8), dimethyl

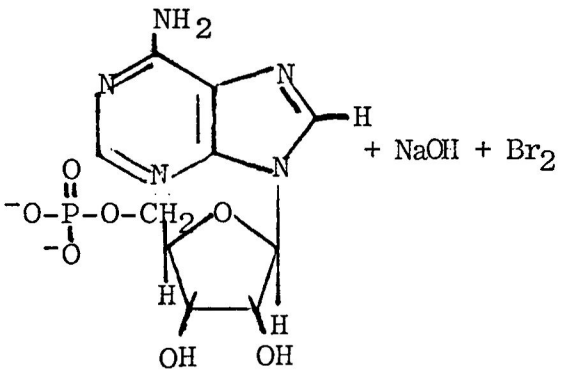
sulfoxide (DMSO), and phosphate buffer (pH 6.8).

RESULTS AND DISCUSSION

Adenosine 5'-monophosphate (AMP) is an allosteric inhibitor of fructose 1,6-bisphosphatase. An allosteric inhibitor is defined as an inhibitor which binds to site(s) of the enzyme other than the catalytic site(s) of the enzyme. In recent years physical techniques such as fluorescence, circular dichroism and optical rotary dispersion spectroscopy have been used to monitor the conformational changes induced by AMP, however the technique of spin-labelling enables the researcher to quantify observed conformational changes.

Adenosine 5'-monophosphate bears a structural similarity to cyclic adenosine 5'-monophosphate. In the initial experiments, cAMP was chosen as a model for the bromination reaction needed for ultimate success in the spin-labelling of AMP.

The product of this first reaction would be 8-bromo-adenosine 5'-monophosphate (25). However, the phosphate group in AMP is not stable in the presence of sodium hydroxide and water. Both systems, AMP and cAMP, are sensitive to the amount of time the heat is applied.



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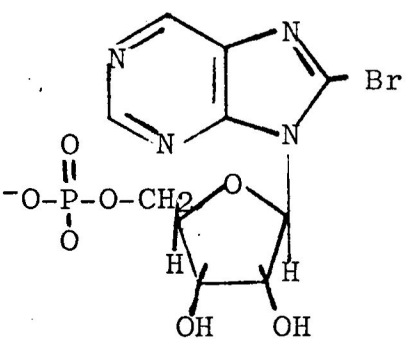
+ NaOH + Br_2



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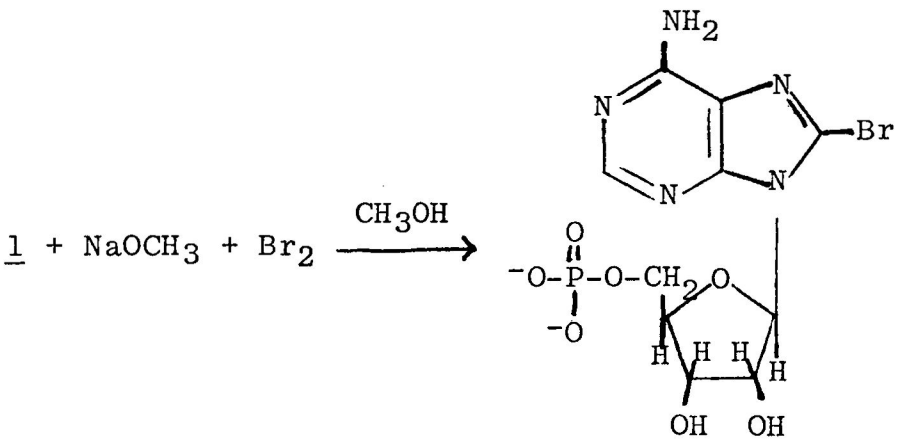
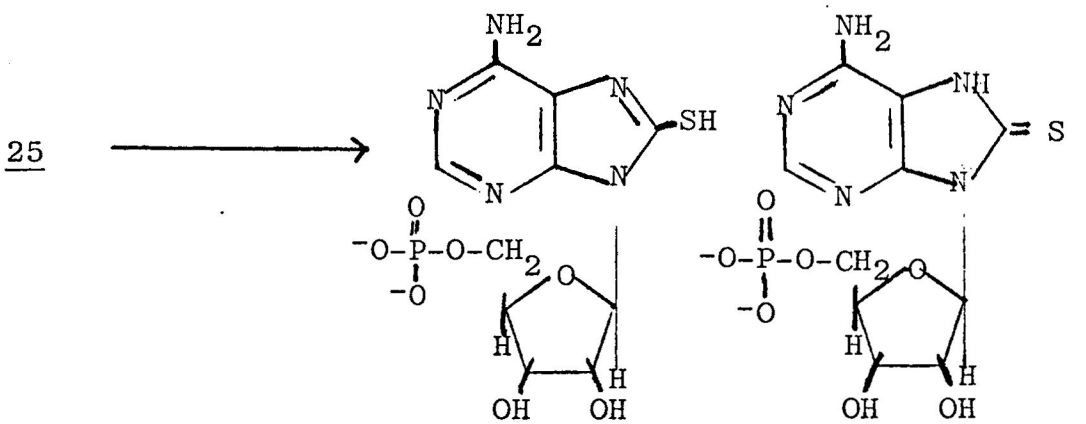
A second procedure to synthesize the 8-bromopurine nucleotide was developed taking into consideration the sensitivity of the phosphate group and the possible destruction of the aromatic nucleus of the purine system. Under conditions of a milder base (sodium methoxide) the possibility of ring opening was minimized. However, cleavage of the phosphate group was still an important consideration.



In 1929 Fiske and Subbarrow[10] developed a very sensitive method for the determination of inorganic phosphates in blood and urine. Fiske and Subbarrow's method is time dependent in that the sulfuric acid component of the reaction mixture (see "Experimental" above) will in time cleave the phosphate group.

Typically, the progress of the reaction was followed by monitoring the disappearance of the absorption bands at 1500 and 1565 cm^{-1} and appearance of an absorption band at 550 cm^{-1}. Upon completion of the reaction (48 hours), the reaction

mixture was placed on a silica gel column and eluted off with methanol and water. The eluent was monitored by ultraviolet spectroscopy at 263 nm for the product which appeared in a broad undefined peak (Fig. 3). The fractions containing the product were placed on a ground charcoal column for further purification (Fig. 4). Tubes containing the product were rotary evaporated to dryness. The residue was collected and stored in a dessicator placed in a refrigerator freezer. Attention was then focused on the step of the synthetic scheme--nucleophilic substitution of the bromo group for a mercapto group (26A), on the purine moiety of 8-bromo AMP.



26A 26B

Nucleophilic substitution of the bromo group in the presence of thiourea with sodium methoxide in methanol yielded a

Fig. 3. Ultraviolet absorption of fractions collected from silica gel column.

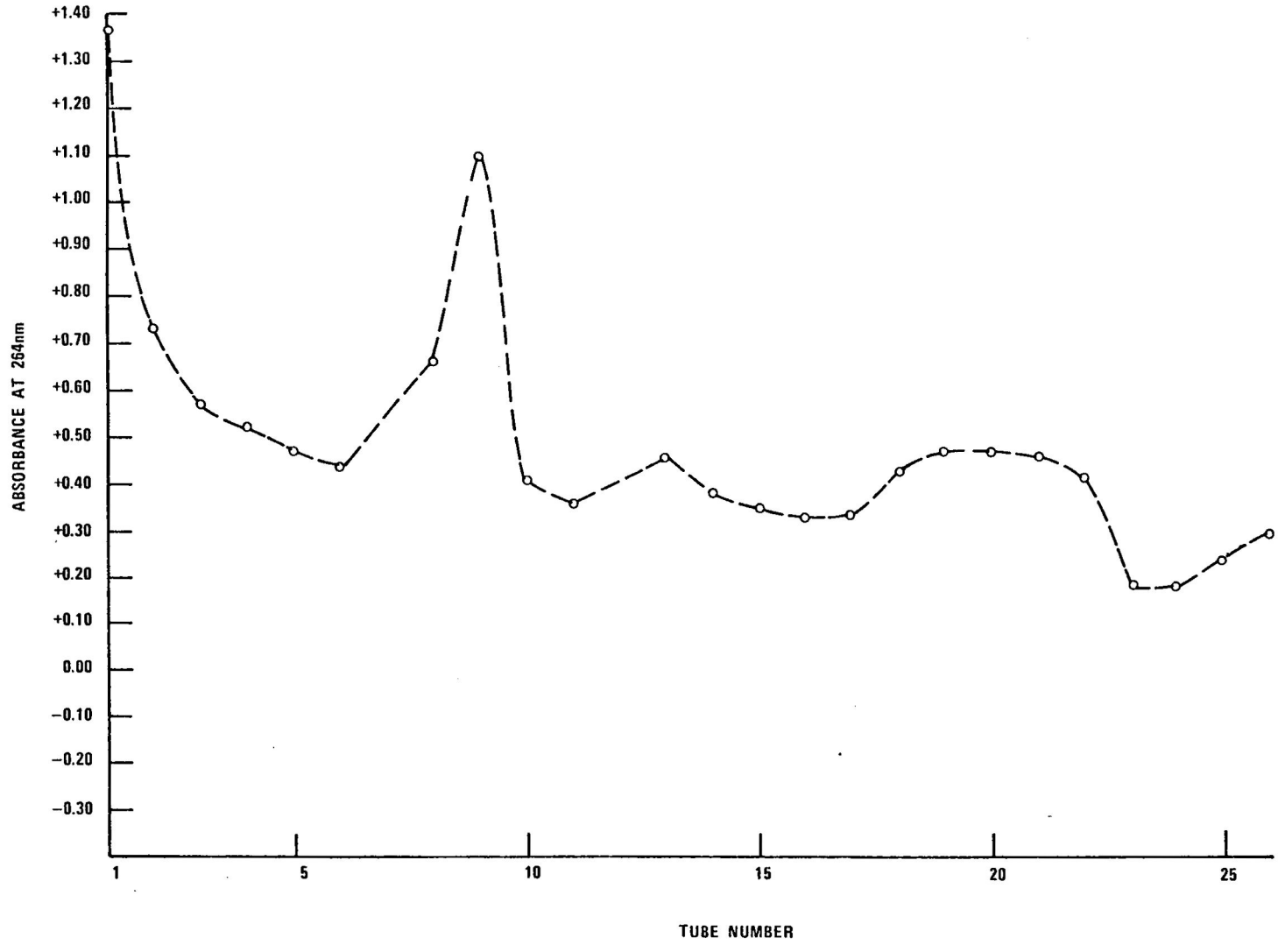
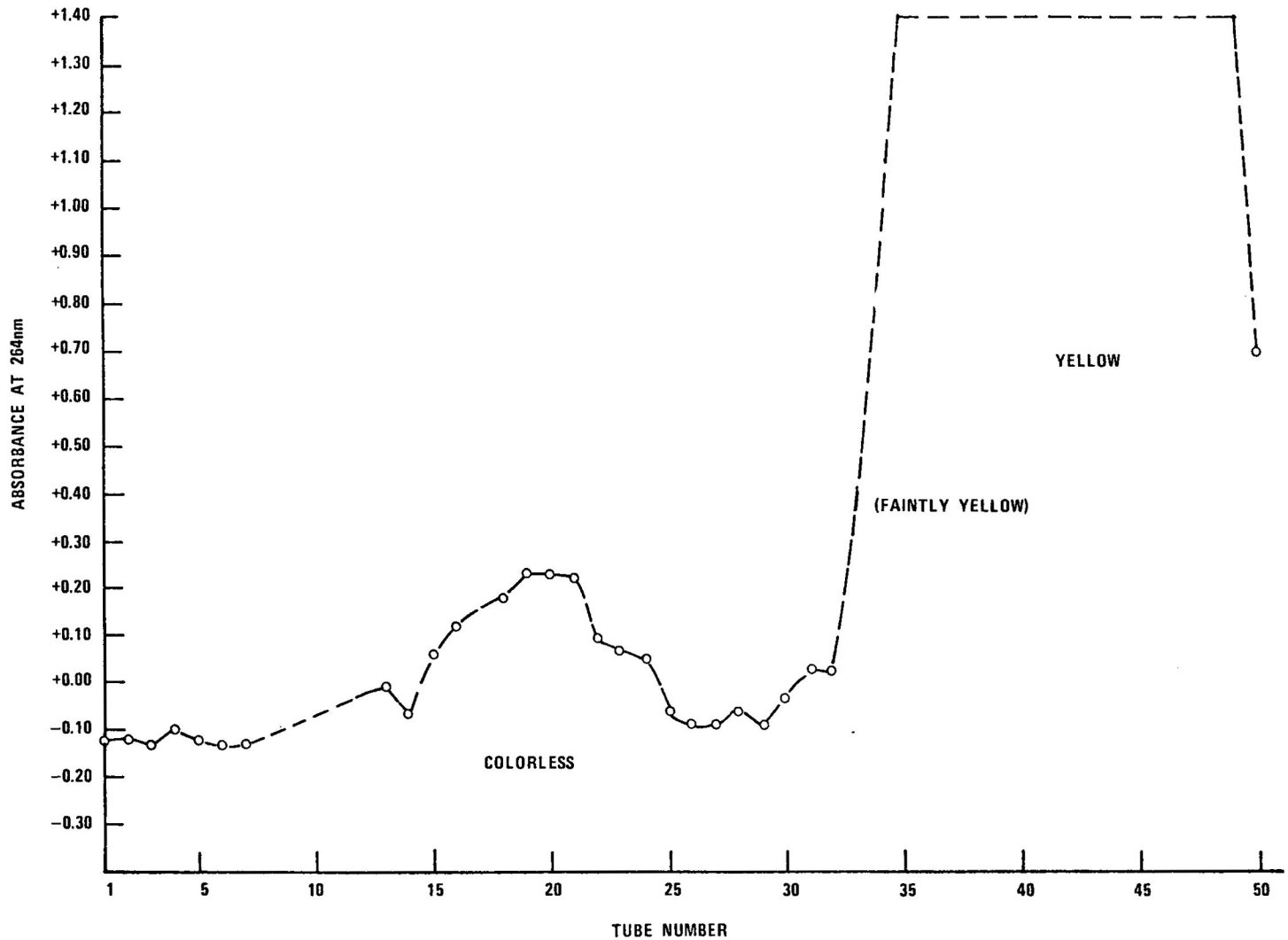

+1.40
+1.30
+1.20
+1.10
+1.00
+0.90
+0.80
+0.70
+0.60
+0.50
+0.40
+0.30
+0.20
+0.10
0.00
−0.10
−0.20
−0.30
ABSORBANCE AT 264nm
1
5
10
15
20
25
TUBE NUMBER

Fig. 4. Ultraviolet absorption of fractions collected from charcoal column.


+1.40
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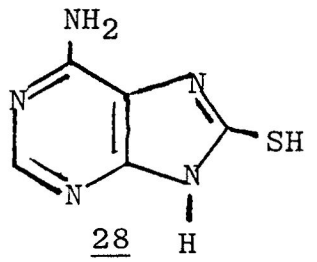
+0.20
+0.10
+0.00
−0.10
−0.20
−0.30
ABSORBANCE AT 264nm
1
5
10
15
20
25
30
35
40
45
50
TUBE NUMBER
COLORLESS
(FAINTLY YELLOW)
YELLOW

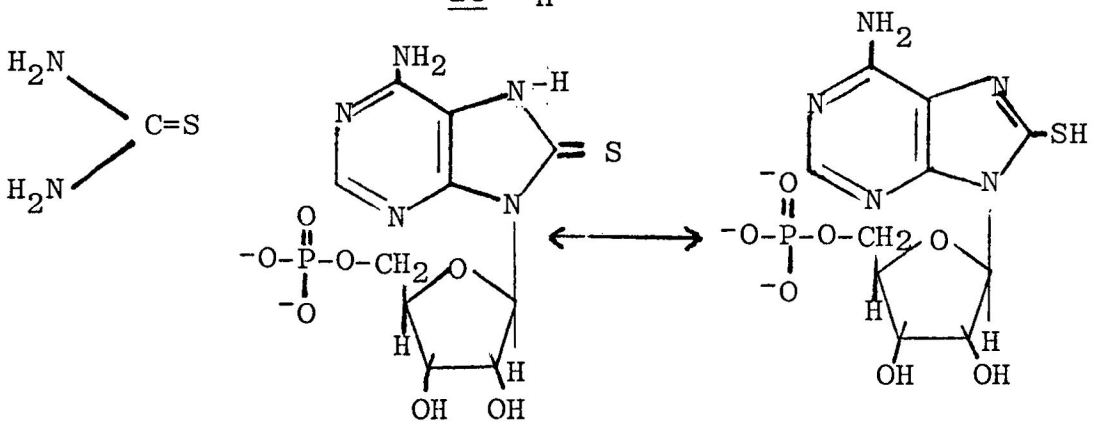
compound that could not be separated by thin layer chromatography. Further investigation using thiourea in ethanol gave similar results as the reaction using sodium methoxide in methanol. Sodium sulfhydrate (NaSH x H_2O) in the presence of dicyclohexylcarbodiimide (DCC) in methanol was allowed to react for two days at 50°. The products were tested by TLC. Dicyclohexylcarbodiimide was separated from the mixture by using a silica gel column and eluted with methanol and water. Infrared spectra indicated the loss of the phosphate group (1680 cm^{-1}). The phosphate loss was confirmed by Fiske-Subbarrow's method. Acetyl-1-thiourea and 1-benzoyl-2-thiourea gave the same infrared results when both were allowed to react for 48 hours at 50°. There was a decrease in absorption in peaks at 1680 cm^{-1} (phosphate group) and 550 cm^{-1} (bromine) and a substantial increase in the peak at 615 cm^{-1} (N-C=S) which supported the presence of sulfur. Since the substituted thiourea reactions did not have complete phosphate loss, the reaction mixture containing sodium sulfhydrate was abandoned.

Reducing the temperature to 35°, 1-benzoyl-2-thiourea and acetyl-1-thiourea gave no evidence of any reaction. Raising the temperature to 42°, the reaction mixture of 1-benzoyl-2-thiourea and 8-bromo-AMP in ethanol was allowed to react for 24 hours. Thin layer chromatography indicated that only one product was formed at the end of 24 hours.

Infrared data verified the presence of the primary amine (3305 and 3125 cm^{-1}), aromatic C-H (2920, 2960, and 2825 cm^{-1}), phosphate group (1680 cm^{-1}) and the absence of bromine (550 cm^{-1}) (Fig. 5). Unlike regular mercaptans, which absorb from 2650 to 2500 cm^{-1}, mercaptopurines absorb like thiocarbonyls (C=S), i.e., around 1520 to 1540 cm^{-1}. For example, 6-amino-8-mercaptopurine (28), easily recognized as the purine moiety of 8-thio-AMP does not have an absorption peak from 2650 to 2500 cm^{-1}, but does have an absorption peak around 1530 cm^{-1}. Ultraviolet spectra were recorded of the new compound (Fig. 6). Two peaks were observed--one at 270 nm due to the nucleotide structure and the other (240 nm) due to the substituted thiourea structure of the thio compound. Thiourea itself absorbs at 241 nm.



28



26A 26B

Fig. 5. Infrared spectrum of 8-mercapto-AMP.

28

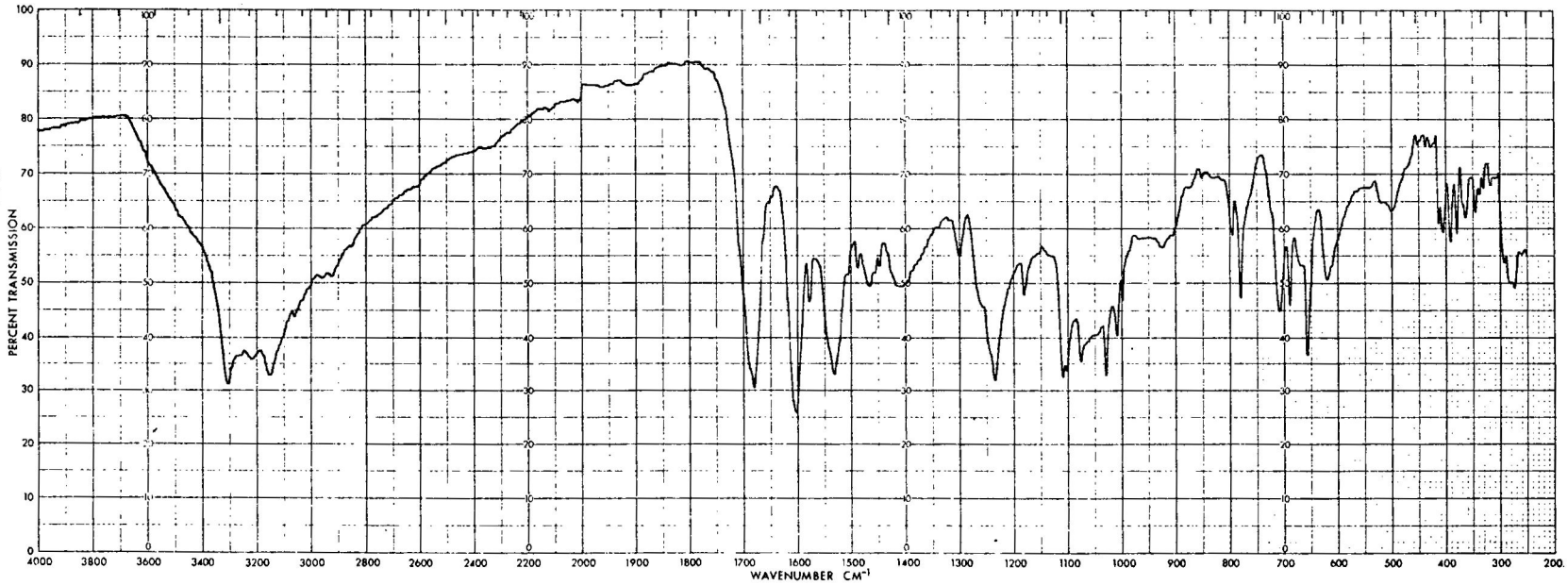
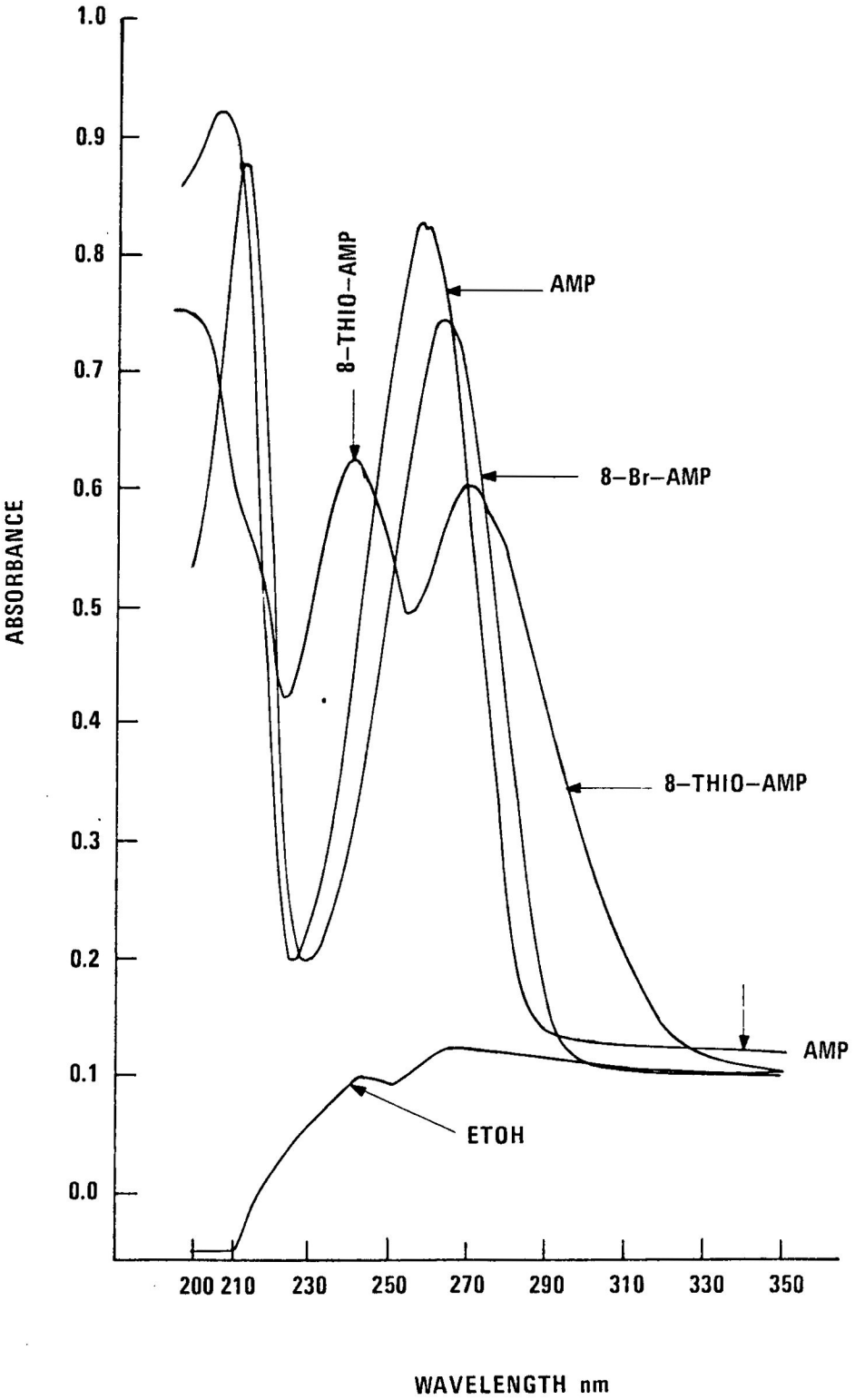
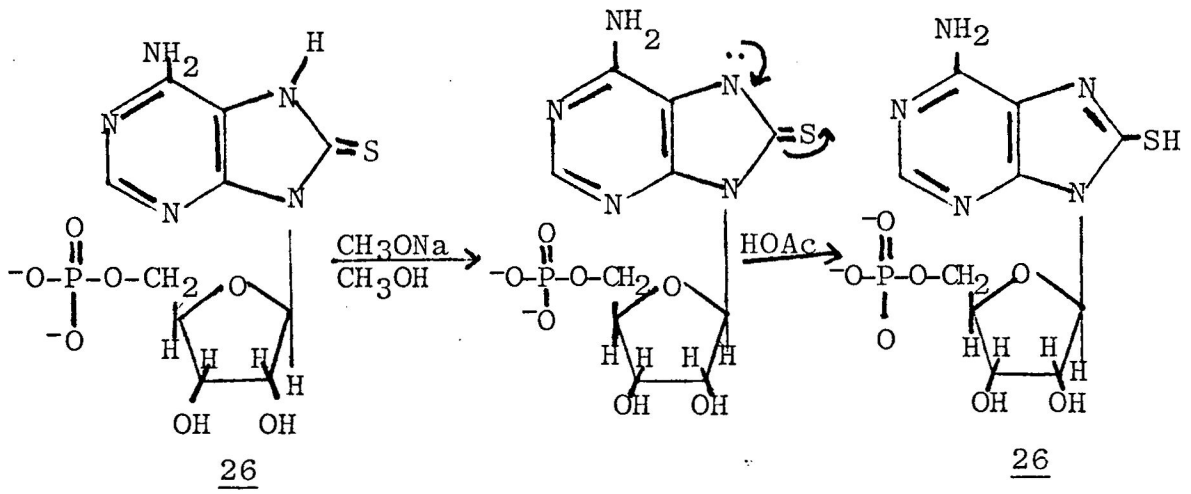

PERCENT TRANSMISSION
100
90
80
70
60
50
40
30
20
10
0
4000 3800 3600 3400 3200 3000 2800 2600 2400 2200 2000 1900 1800 1700 1600 1500 1400 1300 1200 1100 1000 900 800 700 600 500 400 300 200
WAVENUMBER CM⁻¹

Fig. 6. Ultraviolet absorption of 8-thio-AMP.

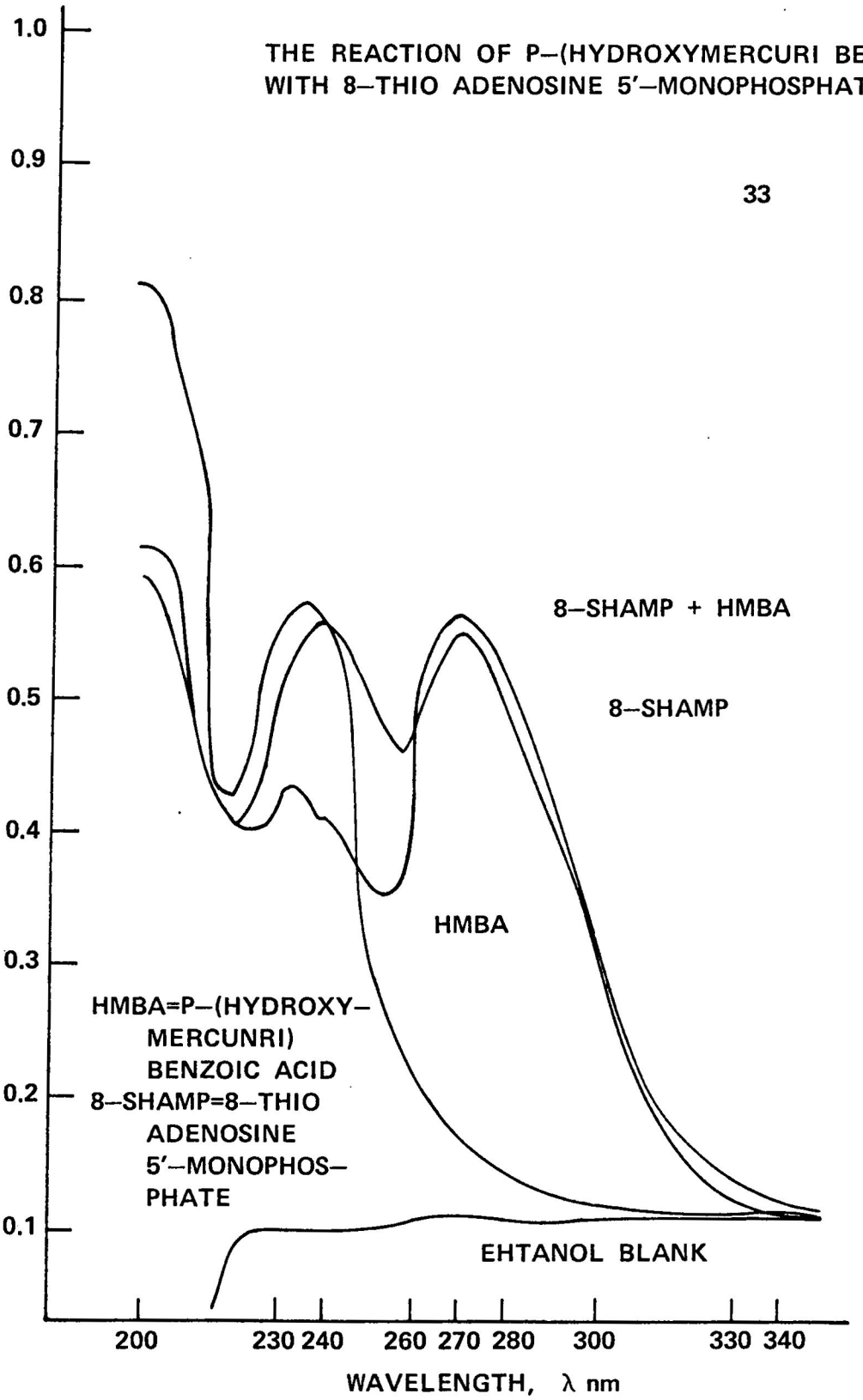

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABSORBANCE
8-THIO-AMP
AMP
8-Br-AMP
8-THIO-AMP
AMP
ETOH
200 210 230 250 270 290 310 330 350
WAVELENGTH nm

The best known test for the identification of sulfur is the Benedict's test.[13] This test, however, failed to give conclusive proof as to the presence of sulfur. In 1968, J. Legett Bailey[14] developed a technique for the identification of mercaptans. In his method the loss of p-(hydroxymercuri)-benzoic acid is calculated from the decrease in absorption and is taken as equivalent to the thiol content of the sample. The results of this test can be seen in Fig. 7. Assuming that the 8-mercapto derivative was in the thiocarbonyl form, an attempt was made to reduce the sulfur. A mechanism to shift the equilibrium to the formation of the mercapto product was developed by Chapp in 1976.[14] Infrared data did not show an absorption band between 2650 and 2500 cm^{-1}. Dithiothreitol was used to keep sulfur in the reduced state.[18] Infrared spectra did not show the mercapto absorption band. No other attempts were made to reduce the sulfur compound. In subsequent sulfur analysis 6-amino-8-mercaptopurine (28) was used as the model.



26 26

Fig. 7. Ultraviolet absorption for the determination of sulfur.


THE REACTION OF P–(HYDROXYMERCURI BENZOIC ACID WITH 8–THIO ADENOSINE 5'–MONOPHOSPHATE)
33
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
8–SHAMP + HMBA
8–SHAMP
HMBA
HMBA=P–(HYDROXY–MERCUNRI) BENZOIC ACID
8–SHAMP=8–THIO ADENOSINE 5'–MONOPHOS–PHATE
EHTANOL BLANK
200
230 240
260 270 280
300
330 340
WAVELENGTH, λ nm

Assuming that the 8-thio compound was actually in the reduced state (SH), an attempt was made to increase the peak at 240 nm by the technique of freeze drying the sample. Fig. 8 shows the increase in absorption at 240 nm by the technique of freeze drying over rotary evaporation.

The spin-label (3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy) was attached to the 3-thio derivative of AMP. Table 1 below gives the correlation times of the free spin-label and the spin-labelled nucleotide in various solvents (Fig. 9).[17]

Table 1. Correlation Times of the Spin-Label and Spin-Labelled Nucleotide in Various Solvents.

SAMPLE	SOLVENT	CORRELATION TIMES LINEAR	QUADRATIC
3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy spin label	DMSO	0.078	0.203
	Tris-HCl Buffer pH 6.8	0.0578	0.0573
	Phosphate Buffer pH 6.8	0.0539	0.066
Spin-Labelled Nucleotide	DMSO	0.0948	0.0746
	Tris-HCl Buffer pH 6.8	0.0168	0.0611
	Phosphate Buffer pH 6.8	0.08323	0.1649

Fig. 8. Ultraviolet absorption of 8-thio AMP: Freeze dried vs. rotary evaporation.

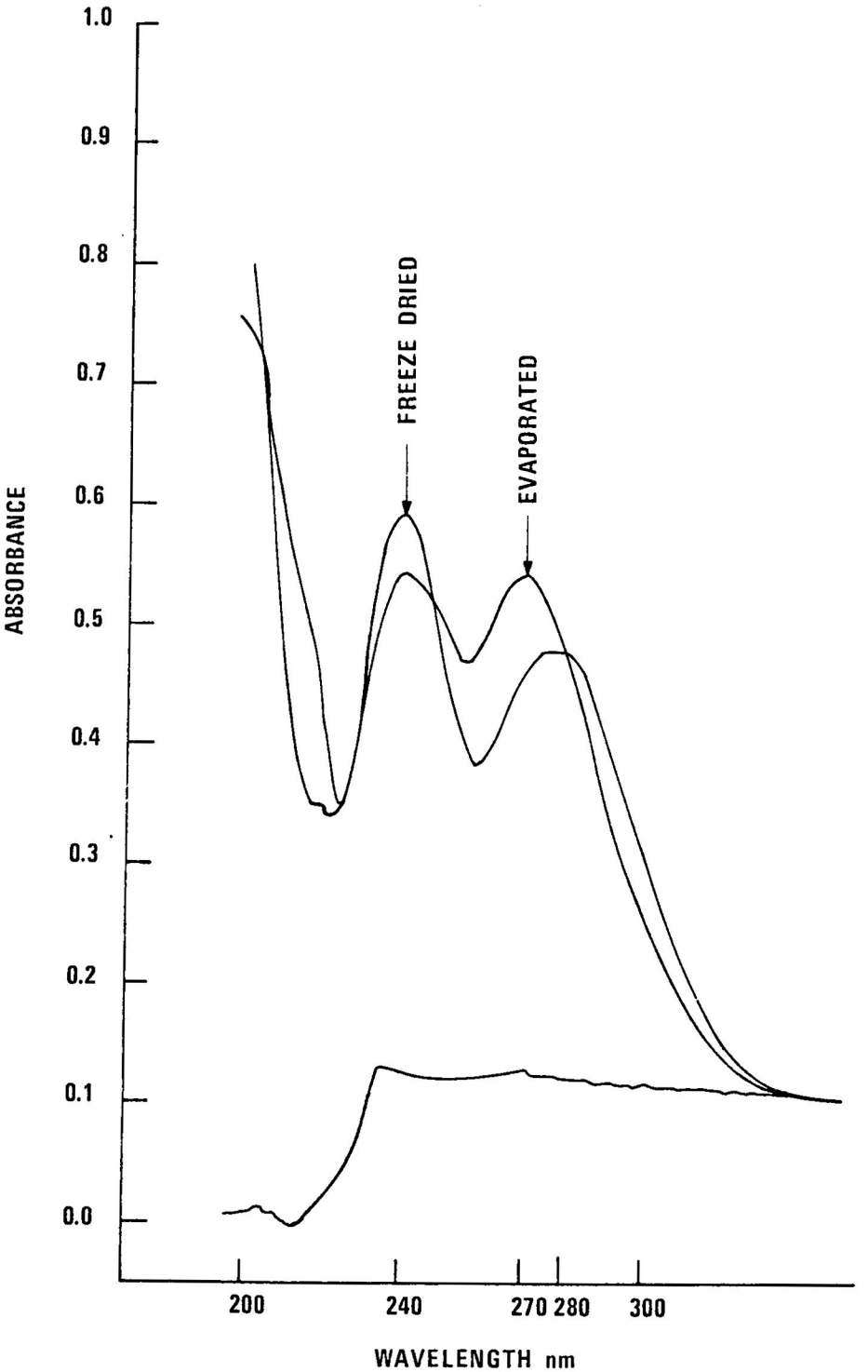
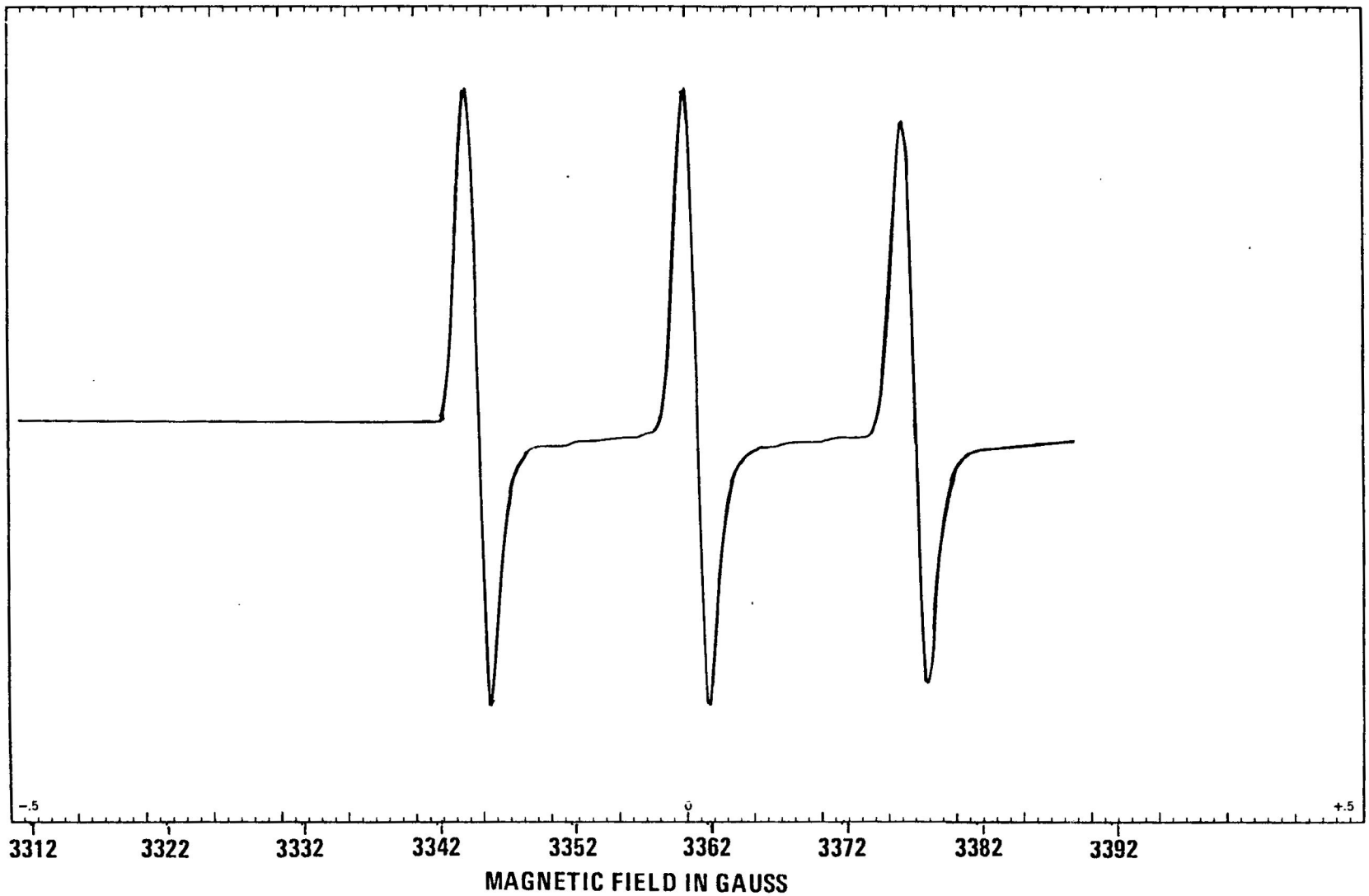

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABSORBANCE
FREEZE DRIED
EVAPORATED
200
240
270 280
300
WAVELENGTH nm

Fig. 9. The ESR spectrum of the free spin-label 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy in Tris-HCl (pH 6.8).


-.5
0
+.5
3312
3322
3332
3342
3352
3362
3372
3382
3392
MAGNETIC FIELD IN GAUSS

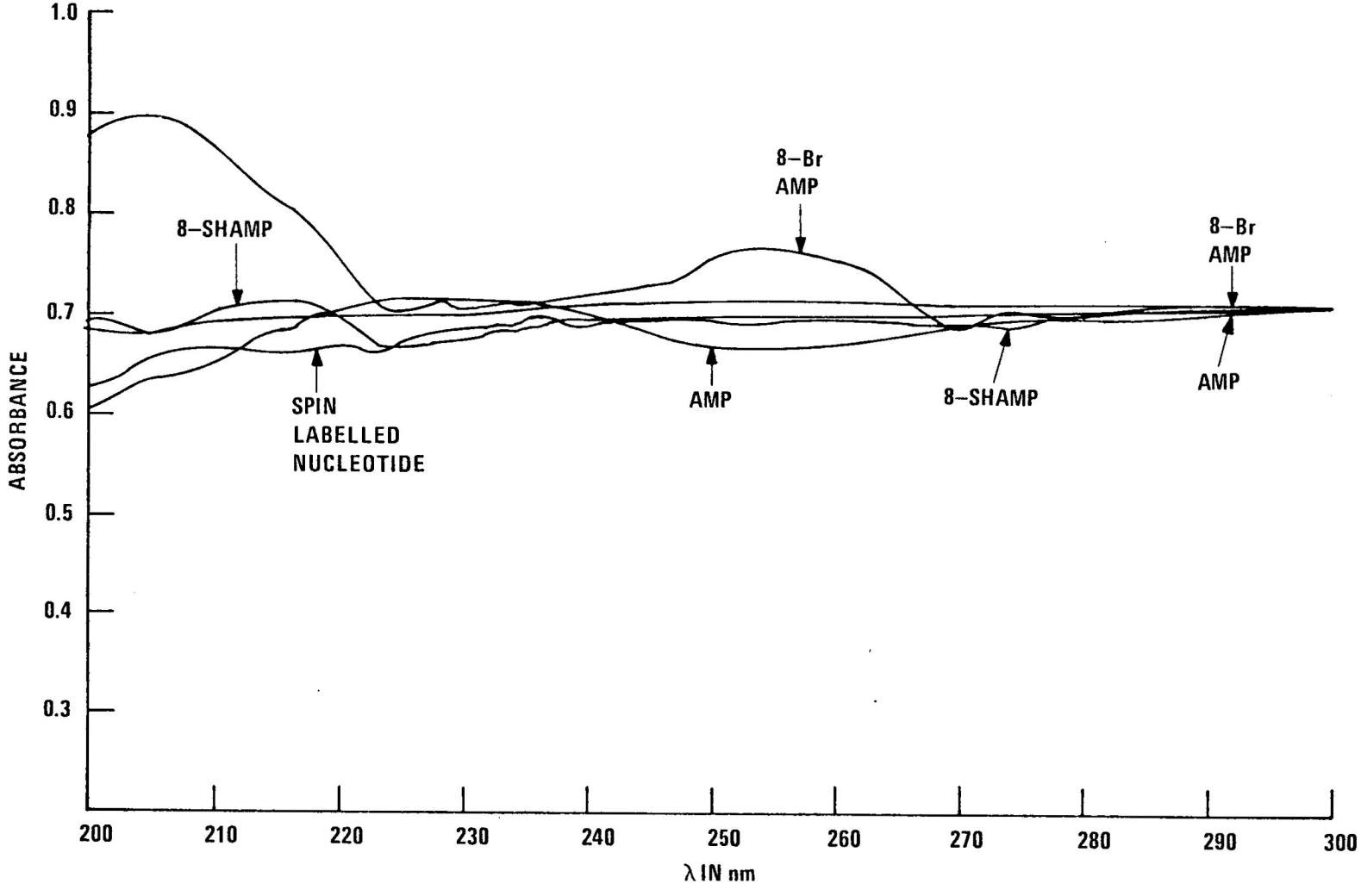
Subsequent ESR studies on the interaction of the spin-labelled nucleotide with AMP specific enzymes, like FbPase, require knowledge about the affinity of different derivatives. In the case of FbPase this can be measured by the difference in rate per unit time (activity) of FbPase with AMP, 8-thio-AMP, 8-bromo-AMP, and the spin-labelled nucleotide using the method of Han.[14] The results of this study can be seen in Table 2. The data in Table 2 show that substitution at C-8 significantly affects the interaction affinity of FbPase for the AMP derivatives.

Table 2. Effects of AMP Derivatives on FbPase.

COMPOUND	RESIDUAL ACTIVITY (%)
FbPase	100
AMP	18
8-SH-AMP	100
8-Br-AMP	100
Spin-Labelled Nucleotide	100

Circular dichroism spectra of AMP, 8-bromo-AMP, 8-thio-AMP, and the spin-labelled nucleotide in methanol are shown in Fig. 10. The spectra of the unsubstituted AMP shows negative effects at the position of its UV absorption band (263 nm). The 8-bromo AMP derivative shows positive effects

Fig. 10. Circular Dichroism spectrum of AMP, 8-thio AMP, 8-bromo AMP, and spin-labelled nucleotide.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
ABSORBANCE
8–SHAMP
8–Br
AMP
8–Br
AMP
SPIN
LABELLED
NUCLEOTIDE
AMP
8–SHAMP
AMP
200
210
220
230
240
250
260
270
280
290
300
λ IN nm

from 266 to 200 nm. The 8-thio-and spin-labelled derivatives show similar CD spectra in the long-wavelength region. From 220 to 210 nm, the 8-thio-derivative shows positive effects whereas the spin-labelled nucleotide shows negative effects in the same region. According to the work done by Hoppe and Wagner,[6] CD spectra may be interpreted to support the supposition that AMP exists in aqueous solution predominantly in the anti- conformation. For steric reasons AMP derivatives bearing bulky substituents at the C-8 position should adopt the syn- conformation.

From the activity studies and the CD spectra, it can be concluded that the C-8 position is important to the binding of AMP to FbPase.

CONCLUSION

Procedures have been developed for direct bromination of adenosine 5'-monophosphate to yield the 8-bromoadenosine 5'-monophosphate. This 8-bromo nucleotide was treated with 1-benzoyl-2-thiourea to provide 8-mercaptoadenosine 5'-monophosphate. The C-8 spin-labelled derivative of AMP was synthesized by reacting 8-thio-AMP with 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy spin-label.

Circular dichroic studies indicate that substitution at C-8 markedly influences the conformational equilibrium in AMP. In order to determine the affinity of the AMP derivatives for FbPase, activity studies were performed on the compounds. The results show that bulky groups decrease the affinity of the enzyme for the AMP derivatives.

Electron spin resonance spectra were recorded for the spin-labelled nucleotide and free spin-label. Correlation times were calculated for the free spin-label and the spin-labelled nucleotide in various solvents.

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